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MINIREVIEW

Regulatory Responses of the Adaptive Response to Alkylation Damage: a Simple Regulon with Complex Regulatory Features

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Alkylation damage to DNA occurs when cells encounter alkylating agents in the environment or when cellular metabolism produces active alkylators. To cope with DNA alkylation, cells have evolved genes that encode proteins with alkylation-specific DNA repair activities. In *Escherichia coli*, the main response specific for alkylation damage has been called the adaptive response (53). The adaptive response genes are induced upon exposure to exogenous alkylators by Ada-dependent induction, and also during stationary phase by *rpoS*-dependent gene expression, possibly to prevent accumulation of DNA damage due to increased endogenous production of alkylating agents. Recent studies of the regulatory mechanisms of Ada protein and the various responses of the individual promoters regulated by this protein has revealed a complexity of regulation not initially recognized. In this review we describe the roles of the Ada-regulated genes and the regulatory mechanisms that activate gene expression from the three Ada-dependent promoters. We will focus on Ada-dependent induction of the adaptive response genes, fine tuning of individual gene expression according to the growth phase, and the role played by Ada in shutting off the adaptive response.

Ada-DEPENDENT REGULATION OF THE ADAPTIVE RESPONSE GENES

The adaptive response set of genes is comprised of the *ada*, *alkA*, *alkB*, and *aidB* genes. Expression of these genes is regulated by Ada, and their induction provides protection against alkylation damage to DNA. The *ada* gene product has both repair and regulatory activities. These two activities are closely tied to one another, as the Ada protein must be activated to perform its regulatory function and activation is a consequence of its DNA repair activity. Ada has two active methyl acceptor cysteine residues, Cys-69 and Cys-321, that are required for demethylation of DNA. Both sites can become methylated when Ada protein transfers the methyl group from the appropriate substrate DNA lesions to itself. This reaction is irreversible, and methylated Ada (^{me}Ada) is the terminal end product of the demethylation reaction (31). The two methyl acceptor sites present in Ada differ with respect to the lesions repaired. Cys-321 is the methyl acceptor site required for the removal of methyl groups from either O^6-methylguanine or O^4-methylthymine, two highly mutagenic lesions (10, 11). Cys-69 is required for demethylation of phosphomethyltriesters in the sugar-phosphate backbone. This lesion is apparently innocuous, since Ada repairs only one of two stereoisomers (16, 36, 37, 72), leaving the other to remain in DNA with no apparent deleterious consequences (28, 40). Although methylated phosphates are innocuous, this lesion is readily produced by methylating agents (37) and provides a sensitive regulatory signal that leads to induction of the Ada regulon. Once Ada protein transfers a methyl group from the methyl-phosphate to the Cys-69 residue, it becomes a transcriptional activator. Thus, the methylated phosphates in DNA serve as the signal that converts Ada to its transcriptionally active form, which, in turn, induces the Ada regulon, resulting in increased alkylation repair activities. The adaptive response genes are induced most effectively by methylating agents and are either not induced or induced only weakly by larger alkyl groups (67), presumably because alkyl lesions larger than methyl groups are efficiently repaired by the *uvrABCD*-dependent nucleotide excision repair pathway and are poor substrates for the adaptive response repair genes (65, 70).

ROLES OF *ada*-REGULATED GENES

The functions of the *ada*-regulated genes have been the subject of several reviews (32, 56, 58, 66) and will be only briefly discussed here. The *ada* gene, described above, is in an operon with the *alkB* gene, and transcription of both genes is directed by the *ada* promoter. The enzymatic function of *alkB* remains elusive despite numerous efforts to determine its biochemical function (8, 71). *alkB* mutants are hypersensitive to the methylating agent methyl methanesulfonate (MMS) and dimethyl sulfate, showing only modest sensitivity to *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine and methyl nitrosourea (5, 17, 68). Because *alkB* mutants were deficient in their ability to reactivate MMS-treated λ phage, which implies that AlkB is able to repair lesions introduced into phage DNA prior to infection, AlkB has been implicated as a DNA repair protein (17). More recently it has been demonstrated that *alkB* is required for reactivation of MMS-treated single-stranded phage. Since no lesions appear to be removed in this process, it has been suggested that *alkB* is involved in replication of damaged template DNA (9). Regardless of its precise function, the fact that *alkB* expression can confer MMS resistance when expressed in mammalian cells suggests it functions by itself (5). The *alkA* gene encodes a glycosylase that repairs a variety of lesions including N^7-methylguanine and N^3-methyl purines and

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O^2-methyl pyrimidines (32). The AlkA protein removes a damaged base from the sugar-phosphate backbone by cleaving the glycosylic bond attaching the base to the sugar, producing an abasic site. Further processing of the abasic site by AP endonucleases, polymerase I, and ligase then completes the repair (32, 66). The function of the *aidB* gene has not been conclusively established, but it is homologous to the mammalian isovaleryl coenzyme A dehydrogenase (IVD), has IVD activity, and appears to function to inactivate nitrosoguanidines or their reactive intermediates produced during metabolic detoxification (25).

MECHANISM OF TRANSCRIPTION ACTIVATION BY ^{me}Ada

According to the model for ^{me}Ada activation proposed by Sakumi et al. (52), ^{me}Ada contacts RNA polymerase through protein-protein interaction with the C-terminal domain (CTD) of its α subunit (αCTD) and recruits RNA polymerase to the Ada-dependent promoters. The evidence for this model was based on the observation that truncations in αCTD abolish transcription from the *ada* promoter in the presence of ^{me}Ada. However, more recent data indicate that RNA polymerase binds to the −60 to −40 region of the *ada* and *aidB* promoters via its αCTD, regardless of the presence of Ada (26). This region serves as the ^{me}Ada binding site, but it also closely resembles, in A+T content, location, and function, the UP element transcription enhancer sequence identified in the *rrnB*P1 promoter (49). At this promoter, truncation of RNA polymerase αCTD, or substitution of its R265 residue by alanine, abolishes α binding to the UP element (14). These mutations also affect RNA polymerase binding to the −60 to −40 region of *ada* and *aidB* and impair transcription initiation (26). Based on these observations, the −60 to −40 regions of *ada* and *aidB* are functionally similar to the *rrnB* UP element and they are sufficient to recruit RNA polymerase. However, only upon binding of ^{me}Ada does the formation of a ternary complex that is proficient in transcription initiation take place (26). Thus, substitutions in αCTD result in reduced transcription from *ada*-dependent promoters because they prevent α from binding the promoter −60 to −40 transcription enhancer sequence, rather than impairing its direct interaction with ^{me}Ada.

Several lines of evidence clearly indicate that the target in RNA polymerase for activation by the Ada protein is the σ^{70} subunit. Gel retardation experiments (20) show that direct protein-protein interaction takes place between ^{me}Ada and σ^{70} and that the determinants for such interaction reside in the C-terminal 39 amino acids of σ^{70}. Substitutions of several amino acids in the C-terminal region of σ^{70} impair Ada-dependent transcription both in vivo and in vitro (20, 21), indicating that ^{me}Ada-σ^{70} interaction is indeed necessary for transcription activation.

^{me}Ada-σ^{70} INTERACTION AT THE *ada* AND *aidB* PROMOTERS

At the *ada* and *aidB* promoters, Ada interacts with a negatively charged patch in σ^{70}: with the single exception of residue I590, substitutions affecting Ada-dependent transcription are in negatively charged amino acids (E574, E575, E591, E605, and D612). Two of these residues (E575 and E591) are also involved in the interaction with the activator proteins PhoB and cI, respectively (19, 35), suggesting that these amino acids might be surface exposed and accessible to different activators. In the absence of ^{me}Ada, RNA polymerase can bind to the promoters via its αCTD, but it fails to establish any strong interaction with the core promoter. ^{me}Ada does not recruit RNA polymerase to the *ada* and *aidB* promoters, since binding of Ada and α to the −60 to −40 region is noncooperative (26). Instead, upon binding of α to the UP element, ^{me}Ada interacts with σ^{70}, activating transcription; therefore, α subunit-promoter and ^{me}Ada-σ^{70} interactions act at separate but interdependent steps of transcription initiation. At the *ada* and *aidB* promoters, ^{me}Ada appears either to increase binding to the core promoter region or to favor the formation of the open complex, functions that are indeed typical for activators that interact with the σ subunit of RNA polymerase (29, 48).

As shown in Fig. 1, the Ada protein is structured in two independent domains, linked by a hinge region that is highly susceptible to proteolytic cleavage (7, 63). The N-terminal domain of the Ada protein (AdaNTD) carries the determinants for specific DNA binding: methylation of cysteine-69, the methyl acceptor site for methyl-phosphotriesters, allows AdaNTD to specifically bind DNA. ^{me}AdaNTD binds to the Ada binding site with an affinity similar to that of the full-length protein and protects the same bases in DNase I protection assays. However, ^{me}AdaNTD is not able to activate transcription at the *ada* and *aidB* promoters. Thus, the determinants for interaction with RNA polymerase (i.e., the "activating region" of the Ada protein for *ada* and *aidB*) must reside in the CTD of the Ada protein (1). This is further substantiated by the extensive mutational studies of the AdaCTD performed by Shevell and Walker (57, 59).

The results of recent studies (20) suggest the possibility that the activating region of the Ada protein at *ada* and *aidB* might be a positively charged patch in AdaCTD. Indeed, interaction between surface-exposed patches of opposite electrical charges is a common feature for transcription activation and for protein-protein interactions at large (29, 46–48). Interestingly, the methylation acceptor sites of Ada are part of two distinct positively charged patches: cysteine-69, (AdaNTD methylation site) is part of a PCKR amino acid sequence, while cysteine-321 (AdaCTD methylation site) is located in the similar sequence PCHR. The presence of positively charged amino acids in the proximity of the cysteine residues that function as methyl acceptor sites was proposed to be important for interaction with DNA and DNA repair activity of the Ada protein (42).

Structural data show that cysteine-321 and the flanking amino acids are buried inside the protein and are not accessible to solvents in the unmethylated Ada protein. However, upon DNA binding, the PCHR patch becomes exposed at the surface of the protein; methylation of cysteine-321 stabilizes this conformation (42). Methylation of cysteine-321 is necessary for optimal activation of *ada* transcription (61, 64), which is consistent with a possible involvement of the PCHR motif in Ada-σ^{70} interaction. Interestingly, substitution of cysteine-321 to an alanine results in the exposure of the histidine and arginine residues on the surface of the Ada protein, thus mimicking the effects of cysteine-321 methylation. The C321A mutation of Ada protein results in constitutive activation of the *ada* promoter (60, 61), again suggesting a direct role of the methylation site in the AdaCTD in transcription activation. Alternatively, it is possible that the role of AdaCTD methylation is indirect, triggering a conformational change required to expose an activating region. Such a role would be consistent with the results of Shevell and Walker (57), who reported that truncations of the terminal 20 to 30% of the AdaCTD result in constitutive activation of the *ada* promoter. The truncated proteins are missing the Cys-321 region, and its deletion may expose the interaction domain, allowing it to contact the σ^{70} subunit. However, further deletions in the AdaCTD abolish activation at the *ada* promoter, providing additional evidence

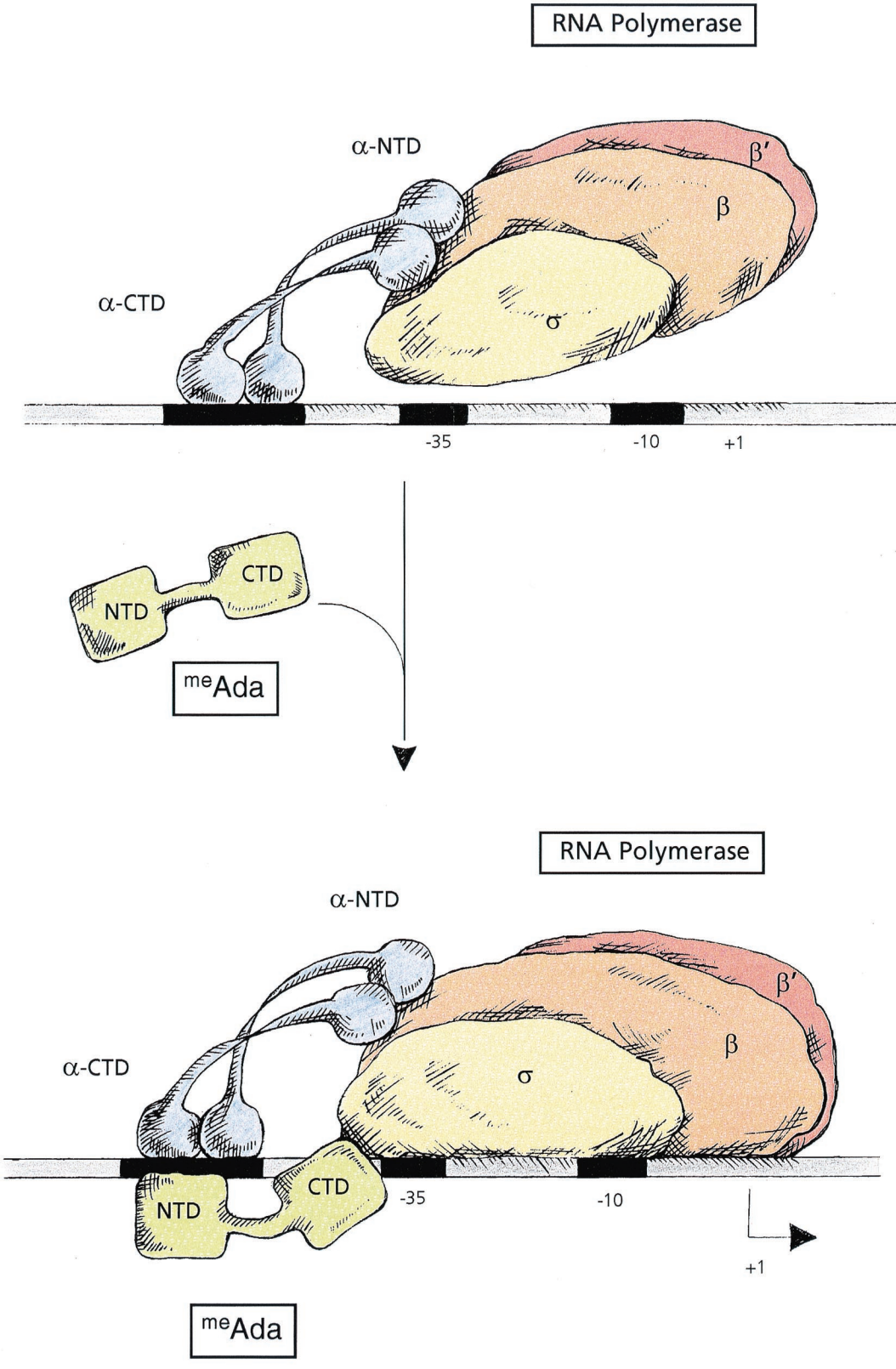

FIG. 1. Model for transcription activation by ^{me}Ada at the *ada* and *aidB* promoters. The upper panel illustrates the specific interactions established between RNA polymerase and promoter DNA in the absence of ^{me}Ada; the RNA polymerase-promoter complex results from protein-DNA interactions between αCTD and the UP elements. The lower panel shows the RNA polymerase-promoter-^{me}Ada ternary complex. ^{me}Ada binds to its DNA site via its NTD and stimulates transcription initiation (black arrow) via protein-protein interaction between its own CTD and the CTD of σ^{70}. Additional evidence suggests that RNA polymerase may also make DNA contacts farther upstream, most likely by bending the DNA. However, the nature of these contacts and their possible functions remain to be determined.

that the determinants for Ada interaction with RNA polymerase reside in the CTD of the protein. Thus, conversion of Cys-321 to A, deletion of part of the CTD including the C321 region, and methylation of Cys-321 may all have similar consequences for Ada activation. The truncated Ada proteins that constitutively activate *ada* transcription require additional activation in order to induce *alkA* transcription, again demonstrating that the regulatory regions required for *alkA* and *ada* induction are distinct and separable by mutation.

Ada-σ^{70} INTERACTION AT THE *alkA* PROMOTER

Although the C-terminal region of σ^{70} is also a target for Ada activation at the *alkA* promoter, a different set of amino acids (K593, K597, and R603) is involved (21). In contrast to the σ^{70} residues necessary for ^{me}Ada-dependent transcription at *ada* and *aidB*, the amino acids involved in transcription activation by Ada at the *alkA* promoter are positively charged. The K593, K597, and R603 residues, as well as other neighboring positively charged residues, are also targeted by other activator proteins. Substitution to alanine of any of these residues severely affects transcription activation by the Fnr protein and by cyclic AMP receptor protein (CRP), at the *dmsA* and p*melR*con promoters, respectively, while R596 appears to be the target site for cI (30, 33). Although the three-dimensional structure of σ^{70} has not yet been solved, two alternative model structures were proposed, based on the highly similar DNA binding regions of the NarL and Cro proteins (3, 41). According to both models, residues K593 and K597 belong to a surface-exposed patch, thus providing an accessible target for activator proteins such as Ada, while R603 is removed from the protein surface and in closer contact with the helix-turn-helix DNA binding motif. The location of the R603 residue would suggest that the RA603 substitution can affect *alkA* transcription by altering the general conformation of the C-terminal region of σ^{70}, consistent with the effects of the RA603 mutation on factor-independent transcription at some promoters (33).

The Ada protein is the first example of a transcription activator able to contact two distinct determinants in the σ^{70} subunit of RNA polymerase in a promoter-specific fashion. Due to the limited flexibility in where the C-terminal region of σ^{70} can be positioned, an activator that interacts with σ^{70} is able to contact its target only if precisely placed. This is in contrast to the situation with activators that interact with αCTD, which, due to its flexible linker, can establish the same kind of interactions with activators at different locations (15, 38). This argues that a σ^{70}-contacting activator that binds at different positions in different promoters must contact alternative determinants in σ^{70}. This is indeed the case for the Ada protein, which binds the *alkA* promoter between −47 and −35, i.e., one helical turn downstream compared to the location of the Ada binding site in *ada* (between −57 and −45) and *aidB* (between −55 and −43). Thus, the use of different targets in σ^{70} appears to depend upon the location of the Ada binding site. The different position of the Ada binding site and the use of a different activation target at the amino acid level would strongly suggest that a different activating region of the Ada protein is responsible for transcription activation at *alkA*. Indeed, in contrast with the *ada* and *aidB* promoters, the unmethylated form of the Ada protein, as well as the methylated form of the AdaNTD, is able to activate transcription at *alkA*, although with a lower efficiency than the full-length methylated Ada protein (1, 43). These observations indicate that methylation of the Ada protein is not required to expose the activating region responsible for *alkA* induction and that these determinants might be located in the AdaNTD. A model contrasting Ada-RNA polymerase promoter interactions at *ada* and *aidB* with the interaction at *alkA* is shown in Fig. 2. Since the CTD of Ada is dispensable for activation at *alkA*, only the NTD is shown to make contact with both α and σ^{70}. However, we cannot rule out the possibility that either α or σ^{70} might establish additional contacts with AdaCTD. Unlike at the *ada* and *aidB* promoters, where ^{me}Ada does not stimulate binding of the αCTD, interaction between the α subunit of RNA polymerase and the Ada protein appears to make an important contribution to transcription activation of the *alkA* promoter (23). It is noteworthy that other transcription activators, such as CRP, Fnr, and the Mor protein, whose binding site is centered around −41, also interact simultaneously with the α and σ^{70} subunits and contact similar activation targets at the amino acid level (2, 33). These proteins are often referred to as ambidextrous activators (45).

EXPRESSION OF THE ADAPTIVE RESPONSE GENES IN STATIONARY PHASE AND Ada-Eσ^{S} INTERACTION

A role for adaptive response genes in stationary phase was suggested first by the fact that cells lacking methyltransferase activity are spontaneous, stationary-phase-specific mutators (34, 44). Recent results indicate that the Ada regulon is indeed induced during stationary phase and protects against active alkylators produced by nitrosation of amino acids in nongrowing cells (55, 62). This form of regulation requires the *rpoS* gene product, which encodes the stationary-phase-specific sigma factor σ^{S}, the key regulatory element required for expression of stationary-phase genes. σ^{S} plays an important role in several stress responses, such as cellular responses to oxidative damage and osmotic shock (4, 27, 39).

^{me}Ada is able to activate transcription by Eσ^{S} as well as Eσ^{70} at both the *ada* and *aidB* promoters (24, 62). These observations are consistent with the fact that the σ^{70} amino acids important for activation by ^{me}Ada at *ada* and *aidB* are also conserved in σ^{S} (20). ^{me}Ada activates *ada* transcription by either Eσ^{70} or Eσ^{S} with roughly the same efficiency, while the lack of a functional *rpoS* gene results in lower expression of the *aidB* gene even in the presence of ^{me}Ada (22, 69), showing that the *aidB* promoter is dependent on both Ada and σ^{S} for optimal expression. In contrast, not only does ^{me}Ada fail to stimulate *alkA* transcription by Eσ^{S}, consistent with the lack of conservation of K593, K597, and R603 in σ^{S}, but it negatively affects Eσ^{S}-dependent transcription both in vivo and in vitro (22). Gel retardation experiments have shown that ^{me}Ada inhibits initial binding of Eσ^{S} to the *alkA* promoter, possibly by competition with Eσ^{S} for the same binding site. It has been shown that, although Eσ^{70} and Eσ^{S} can recognize the same promoter, they differ in the nature of their interaction with the promoter DNA, in particular in the degree of protein-induced DNA bending and possibly in the location of the αCTD in the Eσ^{S}-DNA binary complex (6, 18). ^{me}Ada might bind to a region of the *alkA* promoter important for recognition by Eσ^{S}, but not by Eσ^{70}; alternatively, binding of ^{me}Ada might alter the *alkA* conformation to make it less favorable for interaction with Eσ^{S}.

The negative effect of ^{me}Ada on Eσ^{S}-dependent transcription of *alkA* is likely to have important physiological consequences for shutting off this component of the Ada regulon. Since methylation of the Ada protein is irreversible, the cells need a specific mechanism to turn off transcription of the Ada-dependent genes after the methylation damage of DNA has been repaired. The methylated NTD of Ada as well as the unmethylated form of the protein have been shown to negatively regulate the *ada* promoter and were proposed to be

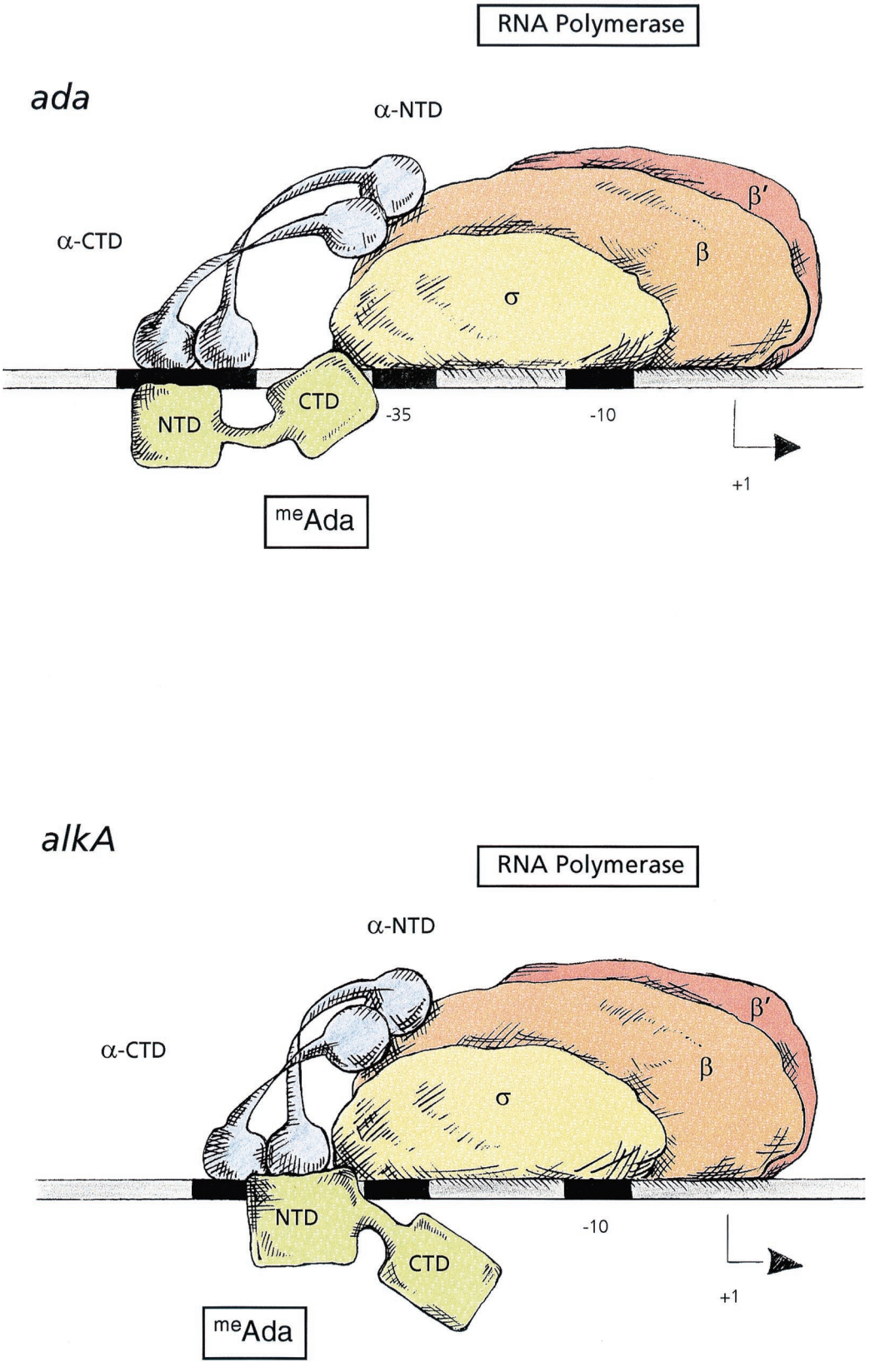

FIG. 2. Model for transcription activation by ^{me}Ada at *ada* and *alkA*. At *ada*, RNA polymerase docks to the promoter region by interactions between the α subunit and DNA, and ^{me}Ada-σ interaction triggers transcription initiation. At *alkA*, ^{me}Ada interacts with both the α and σ subunits, recruiting RNA polymerase to the promoter. Since the CTD of Ada is dispensable for activation at *alkA*, the NTD is shown to be involved in both ^{me}Ada-α and ^{me}Ada-σ contacts.

TABLE 1. Properties of ^{me}Ada-inducible promoters

Promoter	Location of the Ada binding site	Target subunit in RNA polymerase	Effect of ^{me}Ada on transcription by Eσ^S	Activating region in Ada	Regulation by unmethylated Ada
ada	−57 to −45	σ^{70} (negatively charged patch)	Activation	CTD	Repression
aidB	−55 to −43	σ^{70} (negatively charged patch)	Activation (promoter is dependent on Eσ^S for optimal expression)	CTD	???
alkA	−47 to −35	σ^{70} (positively charged patch) and αCTD	Repression	NTD	Activation

involved in shutting off the adaptive response. However, both methylated AdaNTD and unmethylated Ada protein can activate transcription at *alkA*, so that neither mechanism would result in down-regulation of this promoter. Thus, it was postulated that return to low levels of expression from *alkA* occurs by simple dilution of the Ada protein over several growth cycles. The function of ^{me}Ada as a negative regulator of Eσ^S-dependent transcription suggests a specific mechanism for turning off high-level expression of the *alkA* gene: *alkA* expression is activated as long as transcription in the cell is mostly dependent on Eσ^{70}. When cells reach stationary phase, intracellular concentrations of σ^S increase; ^{me}Ada prevents Eσ^S from binding to *alkA*, thus reducing the amount of RNA polymerase available for *alkA* transcription and, in turn, its expression. Similar negative regulation by a functional *rpoS* gene has been observed for another σ^{70}-dependent gene, *uspA* (12), suggesting that σ factors might indeed compete for a limiting amount of RNA polymerase during stationary phase.

Low levels of expression of *alkA* in stationary phase might be tolerated even upon exposure to alkylating agents: the main function of the AlkA protein is removal of 3-methyladenine from DNA. Methylation of this base is toxic because it blocks DNA replication (32). During stationary phase, very little DNA replication takes place, and the need to rapidly repair replication-blocking lesions might be less critical. Thus, the basal levels of *alkA* expression, together with the constitutive expression of the other 3-methyladenine-DNA glycosylase, the Tag protein (44), and with low levels of nucleotide excision repair (65) may be sufficient to repair these lesions, making high-level expression of *alkA* unnecessary.

Interestingly, the adaptive response genes, including *alkA*, are transcribed more efficiently by Eσ^S than by Eσ^{70} both in vitro and in vivo in the absence of ^{me}Ada protein (22, 24). It has been proposed that low concentrations of alkylating agents such as methyl nitrosourea are generated during stationary phase through amino acid nitrosation (62). The observation that strains totally devoid of methyltransferase activity are spontaneous stationary-phase mutators (34, 44) indicates that these proteins are necessary to prevent alkylation mutagenesis. Therefore, an increase in expression of the adaptive response genes in parallel with expression of the genes producing active alkylators during stationary phase prevents alkylation damage to DNA and mutagenesis. It seems more efficient for *Escherichia coli* to counteract methylation damage by endogenously produced alkylating agents by preventing their accumulation, rather than by repairing DNA damage. Indeed, *aidB*, encoding a protein responsible for detoxification of some methylating agents (25, 71), is expressed at an increased rate during stationary phase (23, 67).

CONCLUSIONS

In this report, we have reviewed the mechanisms of transcription activation by the Ada protein. Although only three promoters (*ada*, *aidB*, and *alkA*) are the target of the Ada protein, major differences exist in the mechanisms for their activation. These differences allow fine regulation of the adaptive response genes, which can be differentially expressed according to the specific needs and physiological state of the cell. The main features of transcription activation by Ada are summarized in Table 1.

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